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STAT5 in cancer and immunity

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ABSTRACT

Signal transducers and activators of transcription 5 (STAT5a and STAT5b) are highly homologous proteins that are encoded by two separate genes and are activated by Janus-activated kinases (JAK) downstream of cytokine receptors. STAT5 proteins are activated by a wide variety of hematopoietic and non-hematopoietic cytokines and growth factors, all of which use the JAK-STAT signalling pathway as their main mode of signal transduction. STAT5 proteins critically regulate vital cellular functions such as proliferation, differentiation and survival. The physiological importance of STAT5 proteins is underscored by the plethora of primary human tumours that have aberrant constitutive activation of these proteins, which significantly contributes to tumour cell survival and malignant progression of disease.

STAT5 plays an important role in the maintenance of normal immune function and homeostasis, both of which are regulated by specific members of IL-2 family of cytokines, which share a common gamma chain (γ_c) in their receptor complex. STAT5 critically mediates the biological actions of members of the γ_c family of cytokines in the immune system. Essentially, STAT5 plays a critical role in the function and development of Tregs and consistently activated STAT5 is associated with a suppression in antitumour immunity and an increase in proliferation, invasion and survival of tumour cells. Thus, therapeutic targeting of STAT5 is promising in cancer.

The Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway represents an extremely rapid membrane to nucleus signalling system mediating cytokine signals in mammals [1, 2]. It was studies on the interferon (IFN) receptor signalling that led to the discovery of the JAK-STAT pathway [3]. The JAK–STAT signalling is induced by engagement of a ligand (eg. cytokine) to its corresponding transmembrane receptor. This leads to dimerization of the receptor and thereby activation of receptor-associated JAKs, which subsequently phosphorylate tyrosine residues present in the cytoplasmic domain of the receptor. Phosphorylated tyrosine residues serves as docking sites for the src-homology2 (SH2) domains in STATs. The STATs are then phosphorylated by activated JAKs at a single tyrosine residue in the C terminus. Tyrosine-phosphorylated STATs form homo or heterodimers via reciprocal phosphotyrosine (pTyr)-SH2 interactions and are immediately translocated to the nucleus where they bind to palindromes of the general form TTC(N_{2-4})GAA, termed γ activated sequences (GAS). The STATs are dephosphorylated by nuclear tyrosine phosphatases and exported to the cytoplasm to efficiently continue the phosphorylation-dephosphorylation cycle. Although recent studies on the STAT proteins have demonstrated constitutive energy and transport factor independent shuttling of STATs between the nucleus and cytoplasm [4, 5], it is due to the specific conformation of tyrosine phosphorylated dimers that enables retention in the nucleus and the binding of STzATs to respective GAS sequences [4]. Recent studies have suggested a role for dephosphorylated STAT in the nucleus in maintaining the stability of transcriptionally repressed heterochromatin [6].

STATs and protein structure

The first STATs to be cloned by Darnell and co-workers were the IFN-inducible 91-kDa STAT1and the 113-kDa STAT2 proteins [3, 7]. The remaining five STATs were identified subsequently. There are seven STAT proteins in mammals, STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6, with molecular masses between 75 and 95 kDa and all are mapped to 3 chromosomal clusters being indicative of a common ancestral gene [8]. Out of the seven STATs, STATs 1, 3, 5A, and 5B can be activated by an array of cytokines including growth factors (EGF, PDGF, Hepatocyte growth factor, insulin-like growth factor,

colony stimulating factor–1, Erythropoietin) and hormones (Prolactin, Growth hormone, Insulin) as well as downstream of some G-protein coupled receptors [1, 9, 10]. STATs 2, 4 and 6 are activated by a smaller subset of cytokines (IFN-α, IL-12 and IL-4/IL-13 respectively).

All STAT proteins contain a conserved common structure containing the following domains: the N terminal domain (NTD), coiled-coil domain (CCD), src homology-2 (SH2) domain, linker domain (LD), DNA binding domain (DBD) and a transactivation domain (TAD) at the extreme C-terminus [11]. The conserved N-terminal domain is involved in protein-protein interactions [12], and allows for cooperativity in dimer-dimer interactions and/or to form tetramers that can then bind to non consensus sites [13-15]. The NTD is conserved among the STATs, and it is 91% similar in the human STAT5a and STAT5b. Crystallographic studies on N-terminus of STAT4 revealed that it is composed of eight helices with a hook-like structure, which promote oligomerization of STAT dimers [16] and binding to tandemly-linked GAS sites [14, 17, 18]. Loss of cooperative STAT binding to tandem GAS sequences was observed due to mutation of a conserved tryptophan at position 37 to alanine (W37A), in STAT1, STAT4, STAT5a and STAT5b [15, 16], and furthermore in a loss of cytokine-induced phosphorylation of the critical tyrosine residue in the SH2 domain of STAT4 only [19]. Thus, the NTD serves a common function for all STAT proteins, which is to facilitate cooperative binding to tandemly-linked sites, and a selective role in the receptor-mediated activation of STAT4. Recently, an alternative more thermodynamically stable dimer interface for NTD interactions was determined, which identifies a conserved phenylalanine residue (F77 in STAT1, and F81 in STAT5) as being the critical residue [20]. As with W37A, mutation of this residue in STAT1 and STAT5 leads to a loss of tetramer, but not dimer formation on DNA [4]. Other functions of the NTD include its role in promoting protein-protein interactions such as binding of CBP/p300 to STAT1, receptor domains and PIAS family proteins [19, 21, 22].

The CCD binds to other transcription factors and coactivators [16, 23] and is implicated in nuclear translocation and retention of STAT3 [24] as well as nuclear export of STATs [25]. The DBD, as the name suggests, mediates the binding of STATs to target sites with the canonical GAS sequence $TTCN_{3-4}GAA$ and defines the binding specificity [26-28], although the same is not the case with STAT2 [29]. Immediately following the DBD is a linker domain, which connects the DBD and the SH2 domain. The highly conserved SH2 domain

interacts reciprocally with phosphorylated tyrosine residues on receptors, and thus has a role both in receptor docking and STAT dimerization [11]. The STAT family of proteins are phosphorylated at tyrosine and/or serine residues except STAT2, which is not known to be serine phosphorylated [30-32]. The variable TAD at the C- terminus is critical for transcriptional activation and interacts with additional co-factors [33]. The C-terminus of STAT1, STAT2, STAT5a, STAT5b and STAT6 interact with CBP/p300 [22, 34-36]. Recently it was proved that the DNA replication factor MCM5 which interacts with the C-terminus of STAT1 is essential for STAT1-mediated transcriptional activation [37]. STATs have also been shown to associate with AP1, IRF-1, NF-kB, Sp1, c-Jun, USF-1, Pu.1 and also with the glucocorticoid receptor [38-40].

STAT5

The transcription factor STAT5, was initially identified as a prolactin activated ovine mammary gland factor (MGF) [41]. Studies directed at IL-3 induced signal transduction and cloning, led to the discovery of two highly related isoforms, STAT5a and STAT5b [42, 43]. STAT5a is the major STAT5 isoform in the mammary gland, whereas STAT5b is the major STAT5 isoform in the liver. While STAT5a is mainly responsible for PRL dependent mammary gland development and function [44], STAT5b is required to maintain normal sexually dimorphic GH responses [45]. In humans, the genes for STAT5a and STAT5b map to chromosome 17q11.2, share 91% homology at the amino acid level and differ primarily at their C terminus [8, 42, 43, 46]. Obvious structural differences between the two forms of STAT5 include an additional 12 amino acids on the carboxy-terminus of STAT5a, which gives rise to a slightly shorter STAT5b and a 5 residue abbreviation of the STAT5a phosphotyrosyl tail segment between the SH2 and TAD domains.

Modulation and regulation of STAT5 function

STAT5 can be activated by a diverse group of cytokines, which include prolactin, growth hormone, erythropoietin (Epo), thrombopoietin (Tpo), granulocyte-macrophage colony-stimulating factor (GM-CSF), epidermal growth factor (EGF), IL-2, IL-3, IL-5, IL-7, IL-9 and IL-15 [47, 48] and requires specific kinases (Table 1). The STAT5 signalling pathway is a transient and tightly regulated process, although less is known of the signals leading to its inactivation. The duration of activation of STAT5 is evident within minutes of cytokine

stimulation and disappears a couple of hours later. However, it is found to be constitutively active in numerous primary human tumors, leukemias, and myeloproliferative disorders (MPDs) [49]. STAT5 function is regulated by post-translational modifications, members of SOCS family, PIAS family, caveolins, phosphatases and various protein-protein interactions.

STAT5 signalling is mediated by mechanisms that involve direct and indirect manipulation of STAT5 activity. STATs can be post-translationally modified by acetylation, ubiquitylation, glycosylation, ISGylation, sumoylation and the most common being phosphorylation [50, 51]. Post-translational modification can lead to STAT5 being either positively or negatively regulated.

Tyrosine phosphorylation

STAT5 is activated by tyrosine phosphorylation at position 694 and 699 for STAT5a and STAT5b respectively and can be negatively regulated by dephosphorylation. Tyrosine phosphosphorylation is crucial for STAT5 activity and can be phosphorylated by receptor associated JAKs as well as non-receptor Src kinases. Unlike JAKs, activation of STAT5 by Src kinases, leads to the translocation of only STAT5b into the nucleus [52]. Recent studies suggest a role for the src family of kinases (SFK)-STAT5 pathway in transformations and malignancies [53]. While a number of protein tyrosine kinases have been shown to phosphorylate STAT5, the phosphatases responsible for STAT5 dephosphorylation *in-vivo* remain elusive, despite a number of studies on candidate protein tyrosine phosphatases (PTP), including SHP-1, PTP1B, SHP-2, CD45, T-cell PTP (TC-PTP) and phosphatase 2A, which have been shown to dephosphorylate STATs in vitro [54-58]. Overexpression of PTP1B leads to dephosphorylation of STAT5 [57]. SHP-2 is a STAT5a phosphatase involved in dephosphorylation of the tyrosine-phosphorylated STAT5a in the cytoplasm [20]. Recently it has been shown that the small dual-specificity phosphatase "VHR (Vaccinia H1 Related)" dephosphorylates tyrosine-phosphorylated STAT5, subsequently inhibiting STAT5 function [59]. The phosphorylation of VHR is required to activate its phosphatase activity toward STAT5 [59]. The possibility of other unidentified nuclear phosphatase(s) that dephosphorylates STAT5 still exists. Nevertheless, phosphorylated STAT5 proteins are known to be targeted for degradation via the ubiquitin-mediated proteasomal degradation pathway [60, 61].

Serine phosphorylation

In addition to tyrosine phosphorylation, STAT5a and STAT5b are also induced to undergo serine phosphorylation [1]. Phosphorylation of serine residues is independent of tyrosine phosphorylation. Mutational studies on mouse Stat5 genes and the development of phospho specific antibodies helped to identify serine phosphorylation sites in a conserved PSP motif of Ser725 for STAT5a and Ser730 for STAT5b [62]. Serine phosphorylation at the S725 and S779 residues of STAT5a cooperate to negatively regulate PRL-induced transcription of ß-casein in the absence of costimulation of the glucocorticoid receptor [63]. The biological and physiological implications of STAT5 serine phosphorylation are not completely understood, though a study on expression of p21-activated kinase (Pak1), a serine/threonine protein kinase, showed that it associates with STAT5 and phosphorylates STAT5a at serine position S779 and thereby stimulates ß-casein promoter activity [64]. Clark et al have identified a novel STAT5a serine phosphorylation at S127/128 which is critical for ERBB4-induced STAT5a stimulation and phosphorylation at S779 is regulated by ERBB4 expression in mammary glands [65].

Friedbichler et al in 2010 demonstrated that serine phosphorylation plays an improtant role in leaukemogenesis [66]. More recently, it has been shown that serine phosphorylation leads to nuclear transport of STAT5 in BCR-ABL induced disease (chronic myeloid leukemia) [67].

Glycosylation

STAT5 can be post-translationally modified by glycosylation. A study on HC-11 mammary epithelial cells has reported glycosylation of STAT5a by O-linked N acetyglucosamine (O-GlcNAc) following STAT5 activation and the glycosylated form is mainly found in the nucleus of hormone-induced cells [68]. The glycosylation occurs at the N- terminal threonine T92 position [68]. A mutation at T92 led to loss of STAT5 glycosylation and thereby its ability to bind the transcriptional coactivator CBP. Studies done by Nanashima et al went onto demonstrate that STAT5a was modified by O-GlcNAc in the Hirosaki hairless rat (HHR) and was identified in the nucleus [69]. Additionally, the glycosylated STAT5a was shown to bind to the STAT5-responsive element with an enhanced affinity.

SUMOylation and PIAS

SUMO is an ubiquitin-related molecule and protein-SUMO conjugation (Sumoylation) has been found to have various functions, including positive and negative regulation of STAT transcriptional activity. There are three forms of SUMO peptide, namely SUMO1, SUMO2 and SUMO3, and they consist of around 100 amino acids, which are added to a consensus sumoylation site (ΨKXE). SUMOylation is catalyzed by SUMO-specific E1, E2, and E3 ligases and can be reversed by SUMO-specific proteases (SENPs). Sumoylation has been shown to modify protein function by altering the function, localization and extent of ubiquitination [70]. ICA512 which is a catalytically inactive member of the receptor protein tyrosine phosphatase (PTP) family, mediates the binding of PIASy to STAT5 and sumoylation of ICA512 regulates its binding to STAT5 [71]. Recently, Van-Nguyen et al have shown that the activity of STAT5 is inhibited upon active SUMOylation of STAT5. This was due to an absence of SENP1 and thus, SENP1 played a role in the SUMOylation and regulation of STAT5 transcription during lymphoid development [72].

Originally discovered as negative regulators of STAT signalling, the mammalian protein inhibitors of activated STATs (PIAS) family consists of 5 members, PIAS1, PIAS3, PIASxα, PIASxβ and PIASY [21]. Although several molecular mechanisms have been proposed to explain transcriptional regulation by PIAS proteins, the ones of interest for STAT5 are: PIAS may repress transcription by inhibiting the DNA-binding activity of STAT5 or PIAS may regulate transcription by promoting sumoylation of STAT5. Thus, it was observed by Rycyzyn and Clevenger, that overexpression of PIAS3 in CHO cells represses STAT5 transcriptional activity [73]. Recently, a functional role for the E3 ubiquitin ligase c-Cbl was demonstrated in differentiation of osteoblasts and that blocking c-Cbl activity, downregulates STAT5 ubiquitination and promotes bone regeneration [74].

Transcriptional co-activators of STAT5

A number of proteins are known to interact with STAT family members and interaction of STAT5 with additional proteins including transcription factors and co-activators regulates its activity. Transcriptional initiation requires the interaction of STAT5 with the initiation complex in addition to co-regulatory proteins. These co-activators mainly interact with the

TAD of STAT5, although they are also known to interact via the CCD and SH2 domains. The interaction of STAT5 with other nuclear factors presents a regulatory mechanism that determines greater potency of signalling and/or greater selectivity of target genes [35]. One of the first studied factors was CBP which is the binding protein for cAMP response element binding protein (CREB). CBP and p300 interact with the TAD of STAT5 [75]. Nuclear receptor co-activator 1 (NCoA-1) binds to the TAD domain of STAT5a and is essential for the transcriptional activity of STAT5a [76]. N-myc interacting protein (Nmi) is another STAT5 interacting protein that augments the recruitment of CBP/p300 to STAT5 [35]. The glucocorticoid receptor (GR) has been reported to interact with the N terminus of STAT5 and is required for many functions exerted by either transcription factors [77]. GR binds to STAT5a and has a physiological significance in the expression of milk protein genes [78]. A list of some of the known protein interactions with STAT5 are listed in Table 2.

Interaction of STAT5 to DNA binding sites

The STAT5 proteins interact directly with specific DNA elements through the DNA binding domain in the center of the STAT5 structure along with cooperation of all the other domains. The SH2 domain of STAT plays a vital role in the dimerization [79], and gene regulation of STAT5 may be influenced by the pattern of STAT5a and STAT5b pairing as homo or heterodimers [80-82]. It is known that DNA binding requires dimerization, which is mediated by phosphotyrosyl-SH2 domain interactions between two STAT5 proteins. STAT5 forms supramolecular complexes (dimer-dimer or higher) on target sites containing two or more neighbouring STAT binding sites [4, 15].

STAT dimers can undergo tandem linkage through their N terminal domains when bound to closely spaced GAS sites [17]. STAT5a and STAT5b exhibit differences with respect to their tissue distribution [42, 43]. Park and Waxman in 2001 were able to prove that heterodimerized (STAT5a-STAT5b) and homodimerized (STAT5b-STAT5b) STAT5 complexes play distinct roles in the sexually dimorphic responses of the liver to growth hormone [83]. It has previously been reported that cytokines cause tyrosine phosphorylation and DNA binding of predominantly one STAT5 protein (STAT5a) despite ample expression of both forms of STAT5 in certain cell contexts [84]. One study reported different DNA binding specificities for STAT5a homodimers versus that of STAT5b homodimers due to a single amino acid change in DNA binding domains of the two proteins [85]. However, generally there are no significant differences in DNA binding specificity between STAT5a

and STAT5b [86-88]. The consensus STAT5a and STAT5b binding sites that have been defined *in vitro* are TTCYNRGAA and TTC(T/C)N(G/A)GAA [86, 89]. The STAT5 alignment matrix prepared by Fung et al also favours the consensus GAS motif TTC(T/C)N(G/A)GAA [90].

Previous studies have established a role of the N terminal domain of STAT proteins in formation of tetrameric complexes [16, 18]. STAT5 tetramer formation does not require high affinity sites, and binding of STAT tetramers to weak binding sites produced even more stable complexes than single dimers bound to high affinity sites [15, 16]. Purified STAT5a has a higher DNA binding affinity and could bind to chromatin in the tetrameric form compared to STAT5b which preferentially bound to chromatin as dimers [86]. Tetramer competent STATs could have a wider range of potential DNA binding sites [15, 86]. The importance of tetramerization of STAT5 was shown for the transcriptional activation of CD25 promoter [4, 15]. Tetramerization of STAT5 has been associated with leukemogenesis [91]. A constitutively active STAT5a mutant (cS5F) increased the abundance and stability of STAT5 tetramers compared to the unmutated form and N terminal mutations directed at only tetramer formation failed to induce leukaemia, suggesting that tetrameric STAT5 complexes may regulate a different subset of target genes some of which drive tumorigenesis [91]. A recent study, in STAT5a-Stat5b double knock-in (DKI) mice that form dimers but not tetramers, identified genes regulated by STAT5 tetramers, in addition to defining the consensus sequences required by dimers versus tetramers [92]. The study shows that tetramerization is critical for normal immune function.

BIOLOGICAL FUNCTIONS OF STAT5

Despite sharing ~96% homology at the protein level, both Stat5 proteins have overlapping and distinct functions. Generation of single and double KOs and transgenic mice for STAT5a and Stat5b proteins has greatly enhanced the understanding of the biological roles of these proteins. Indeed, most studies investigationg the role of STAT5 in immunity were performed on the “STAT5 null mice” that still retained a residual protein lacking the N-terminal domain of STAT5, also denoted as the STAT5a/b $^{\Delta N/\Delta N}$ [44, 93, 94]. However, more recent data have been derived from the analysis of STAT5 null mice presenting with a complete deletion of the STAT5a/b gene locus, also denoted as STAT5a/b $^{fl/fl}$, lck-cre and the STAT5a/b $^{null/null}$

mice. The difference between the mouse models STAT5a/b $^{\Delta N/\Delta N}$ and STAT5a/b $^{null/null}$ are strong and implies classified roles for the N terminal truncated STAT5.

Role of STAT5 in immunity

STAT5a/b are crucial regulators of the immune system. An initial understanding of its role came through knock out mice generated in 1998 which was enhanced upon, by the next generation of null mice generated a few years later in 2004 . Thus, the implications of the role of STAT5 are derived from two generations of STAT5 $^{\Delta N}$ /null mice [94, 95].

Studies from the first generation of KO mice (STAT5a/b $^{\Delta N/\Delta N}$)

Most studies that investigated the role of STAT5A/B in lymphopoiesis employed the STAT5A/$^{\Delta N}$, STAT5B/$^{\Delta N}$ and the STAT5A/B$^{\Delta N/\Delta N}$ mice. These STAT5A/$^{\Delta N}$ and STAT5B/$^{\Delta N}$ mice (as well as STAT5A/B$^{\Delta N/\Delta N}$ mice) were viable and revealed surprisingly mild phenotypes in the development and function of T and B lymphocytes. The phenotype of the STAT5A/B$^{\Delta N/\Delta N}$ mice was expectedly, more severe than either of the single KOs (STAT5A/$^{\Delta N}$ or STAT5B/$^{\Delta N}$) alone, and not only showed reduced proliferation of splenocytes but also splenomegaly along with reduced number of NK cells and activated phenotype of T cells [96].

Upon studying the lymphoid development in STAT5A/B$^{\Delta N/\Delta N}$ double mutant mice, there was a subtle reduction in T and B cell numbers, which was accompanied by a complete lack of natural killer (NK) cells and CD4+CD25+ suppressor T cells.

STAT5A/$^{\Delta N}$ deficient T cells presented a reduction in the expression of cytokine receptor IL-2Rα and this was confirmed by the finding that its expression was upregulated by the cytokine IL-2 [97]. Studies on STAT5A/$^{\Delta N}$ and STAT5B/$^{\Delta N}$ mice demonstrated defects in T cell proliferation and function [98]. While splenocytes from STAT5A/$^{\Delta N}$ mice showed partial impairment in IL-2 mediated proliferation, STAT5B/$^{\Delta N}$ mice showed even more severe defect in proliferation [98, 99]. STAT5A/B$^{\Delta N/\Delta N}$ mice caused impaired proliferation in response to IL-2 and halted cell-cycle progression to mature T cells [94, 96, 100]. Both Stat5 proteins regulate TCR-mediated proliferation of CD4 T cells [96], and a greater defect was observed in STAT5B/$^{\Delta N}$ than in STAT5A/$^{\Delta N}$ mice with regard to reduced number of natural killer (NK) cells, suggesting that STAT5a and STAT5b play an unequal role in normal NK

cell development [96]. NK cells are absent in STAT5A/B$^{\Delta N/\Delta N}$ mice and there is no cytotoxic activity in presence or absence of IL-12 or IL-15 [96]. A role for STAT5 in the survival and differentiation of memory CD8 T cells was confirmed by studies using the STAT5A/$^{\Delta N}$ and STAT5B/$^{\Delta N}$ mice, which had reduced numbers of CD8+ T cells from splenocytes and transgenic expression of STAT5 resulted in an increase in the number of CD8+ T cells [101].

It was studies on allergic late-phase reactions that led Kagami et al to demonstrate that STAT5A/$^{\Delta N}$ and STAT5B/$^{\Delta N}$ mice had reduced CD4+ T cell infiltration along with diminished eosinophil recruitment. This was due to defective STAT5 functions in these mice, leading to a reduction in antigen mediated proliferation of splenocytes and T cells and a subsequent decline in the antigen induced T cell infiltration in the airways [102]. The role of STAT5 in T helper cell differentiation was defined on studies using the STAT5A/$^{\Delta N}$ mice. Th2 cell differentiation from antigen-stimulated splenocytes was significantly decreased in STAT5A/$^{\Delta N}$ mice suggesting that STAT5a regulates T helper cell differentiation. The impairment in Th2 cell differentiation was detected in the presence of high concentrations of IL-4, which was restored upon retrovirus-mediated expression of STAT5b. Although Friedrich et al noted that STAT5b is a mediator of IL-4 induced cell proliferation [103], a role for IL-4 mediated activation of STAT5 was dismissed due to no phosphorylation of STAT5a even in the absence of STAT6. There was also an impairment in the development of Tregs (CD4+CD25+) in the STAT5A/$^{\Delta N}$ mice and a depeletion of the Tregs from spelenocytes of these mice did not impair differentiation into Th1 or Th2 cells. Thus, STAT5a is essential for the differentiation into Th2 and development of Tregs.
Additionally, mice deficient in STAT5a/5b demonstrated reduced numbers of peripheral B cells and of B-cell precursors in the bone marrow [104].

Studies from the second generation of STAT5 KO mice (STAT5a/b $^{null/null}$)

Most recent data have been derived from the analysis of STAT5-null mice, in which the entire STAT5a/5b locus was flanked by LoxP sites and further deleted by the CRE-mediated recombination (using the Cre/Lox technology) [95]. The differences between the two mouse models (STAT5A/B$^{\Delta N/\Delta N}$ and STAT5a/b $^{null/null}$) are strong. For instance, STAT5a/b $^{null/null}$ lack CD8+ T lymphocytes and failed to develop T, B and NK cells [105, 106]. Moreover, B-cell development is abrogated at the pre-pro B cell stage in the bone marrow of STAT5a/b

$^{null/null}$ mice when compared to the previous truncated version of STAT5A/B/$^{\Delta N/\Delta N}$ mice. These studies lead to an important conclusion of the differences between the two generations of KO mice. The truncated versions of STAT5 proteins expressed in the STAT5A/B/$^{\Delta N/\Delta N}$ mice are able to compensate and to a large extent not only resucue B cell development, but also support the development of CD8+ T cells. It is now well known that the truncated versions of STAT5 can bind to a fraction of the STAT5 target genes within the nucleus, thereby activating or repressing them.

The first evidence of the role of STAT5 in CD8+ T cell differentiation came with studies on STAT5b-CA mice which exhibited increased numbers of CD8+ T lymphocytes. This was confirmed when Park et al, proved, that strong TCR signals led to a repression in the IL-7R which subsequently led to a downregulation of CD8 expression [107]. According to the co-receptor model, high affinity TCRs differentiate into CD4+ T cells while low affinity TCR's lead to differentiation and expression of CD8+ T cells. Interestingly, the molecular basis of this CD8 T cell differentiation was due to a dependance on STAT5 signalling and was determined by expressing Runx3, the master regulator for CD8 T cells [108]. Thus, STAT5 signalling is required in mature CD8+ T cells for the expression of the CD8 master regulator Runx3 and CD8 .

It was Zhu et al, who in 2003 first suggested a role for STAT5 in Th2 differentiation, by ectopically expressing activated STAT5[109]. This led to a skewing of the cells from Th1 to Th2 fate. These studies demonstrated that expression of STAT5 ectopically, suppressed Th1 and Th17 differentiation, while promoting Th2 cell differentiation by suppressing the production of IFNy. Thus, both STAT5a and STAT5b regulate Th2 cell differentiation. In brief, IL-2 regulates Th2 cell differentiation by upregulating GATA3 and an upregulation in the IL-4R due to the binding of STAT5 to the promoter of the *IL4r* [110]. IL-2 via STAT5 augments and regulates the expression of IL12rb2 and Tbx21, leading to an enhancement in Th1 responses as well [111].

Previous work on the STAT5A/B$^{\Delta N/\Delta N}$ mice had demonstrated that Treg numbers were reduced in these mice and that activation of STAT5 led to an increase in Tregs. However, further work using the STAT5a/b $^{null/null}$ mice established that conditional deletion of STAT5 in double positive thymocytes, resulted in decreased numbers of CD8^{+} T and a failure to develop into Tregs [105]. Most importantly, none of the surviving tregs had the deleted STAT5 protein, which goes on to support its role in the development of Tregs. This

observation was confirmed using STAT5b-CA mice lacking the IL-2Rb, which suggested that STAT5 played a crucial role in rescuing Treg development in the IL-2Rb negative mice. At the same time, these studies also went onto reveal the binding sites of STAT5 on the Foxp3 promoter, which is master regulator of Tregs [112].

Role in haematological malignancy

STAT5 activation has been found in blood malignancies (HTLV-1 dependent leukaemia, Erythroleukemia, Acute lymphocytic leukaemia (ALL), Acute myelogenous leukaemia (AML), Chronic myelogenous leukaemia (CML), megakaryocytic leukaemia (ML) and Hodgkins lymphoma [113-116] and human tumours (breast, prostate, ovary, head and neck cancers) [117, 118]. STAT5 has also been shown to be constitutively activated in cutaneous lymphomas, while dysregulated expression of a C-terminally truncated form of Stat5 in Sezary Syndrome was associated with a loss of IL-2-induced gene expression [119]. Although both the STAT5 isoforms have been involved in human cancers and tumors, the exact role of each isoform in a specific cancer has not yet been illustrated.

With the prominent role played by STAT5 in the development, differentiation and survival of lymphoid cells, it is no wonder then that it is involved in hematologic malignancies. ALL is a neoplastic disease of both children and adults characterised by acquired genetic alteration, chromosomal alterations and translocations are factors that lead to the diagnosis and prognosis of the disease. Studies on the ABL oncogene demonstrated that the STAT5a/b $^{null/null}$ cells are refractory to transformation by the Abelson oncogenes and subsequently fail to induce lymphoid leukemia, whereas STAT5A/B/$^{\Delta N/\Delta N}$-derived cells are readily transformed [105]. Multi-lineage leukemia was also observed in mice expressing the constitutively active STAT5a/b which was due to the tetramerization capability of STAT5a/b in these mice [91]. Thus, although the truncated version of STAT5 is deficient in the tetramerization domain, they are able to compensate for the loss by binding to oncogenic tyrosine kinases (eg ABL oncogene). Xia et al, demonstrated that the naturally occurring truncated forms of STAT5 were responsible for blood cell cancers and was the main cause for the relapse of the cancer [120]. The role of STAT5 in ALL was confirmed when, Nakayama et al, found that STAT5b-CA mice developed symptoms closely related to human pre-B ALL [121]. Another set of studies also demonstrated that a transgenic STAT5-CA

mouse presented with loss of p53 and concurrent initiation of B cell leukemia [122]. Lessons learnt from a limited study of human samples also supported the evidence and that STAT5 was constitutively activated in more than 60% of frozen ALL samples [123, 124].

A classic example of the complex nature of the cascade of signalling pathways that can be activated by a single oncogenic protein in cancers is that of signalling mediated through the Bcr-Abl tyrosine kinase, which also induces constitutive STAT5 activation in CML [117]. Studies on mice suggested that Stat5 was not required for the induction of BCR-ABL-induced CML-like leukemia [104]. In contrast, recent studies showed that STAT5 is required for the development of leukemia upon introduction of BCR-Abl [105]. In the absence of STAT5a, the incidence of CML was reduced [125]. Serine phosphorylation of STAT5 is essential for leukemogenesis and serine phosphorylation of STAT5a is necessary for nuclear localization of STAT5 in BCR-Abl$^+$ cells and that formation of tetramers rather than dimers is associated with leukemogenesis [66, 91]. Furthermore, a recent study demonstrated that BCR-ABL1 aftects STAT5A/B differentially. Using a BCR-ABL positive cell line, STAT5B RNAi knock down led to sensitization of leukemic cells to treatment by imatinib [126], and STAT5A attenuation enhanced the basal oxidative stress and DNA damage of normal CD34 positive and CML cells. Attenuation of STAT5a also resulted in the inhibition of growth in CD34 positive CML cells from imatinib resistant patients [127].

CML is a clonal hematopoietic stem cell (HSC) malignancy characterized by chromosomal translocation and the hallmark oncogenic event is the formation of the mutant BCR-ABL and activation of STAT5 [115]. Indeed, BCR-ABL1 is crucial for the survival and proliferation of leukemic cells in chronic stage CML and Hochhaus et al showed that BCR-ABL1-targeting drugs have posititive response in many patients with Philadelphia chromosome positive (Ph+) CML [128]. Imatinib was one of the most successful drugs with therapeutic implications in leukemia. Dasatinib is another drug with huge implications in leukemia.

Onishi et al identified and characterized a constitutively active STAT5 mutant that induces certain properties of malignant cells [129] and studies have identified the cause of the constitutive activation as being dependent on its specific role in a specific malignancy. Interestingly, constitutive activation of STAT5 is at times due to its induction by constitutively active JAK2. Indeed, the constitutive activation of STAT5 in some

myeloproliferative disorders is due to the JAK2 V617F mutation [130, 131]. And constitutive activation of STAT5 is also observed in myelodysplastic syndrome (MDS) and in myeloproliferative diseases, such as polycythemia vera (PV), where there is associated dysregulation of the upstream activating kinases, JAK1 or JAK2 [132, 133]. However, in AML, constitutive activation of STAT5 is driven by FLT3. *FLT-3* and *c-KIT* gene mutations lead to STAT5 activation in leukemias as noted in these studies [134, 135]. Thus, activated FLT3 has the potential to transform hematopoietic cells and activates STAT5 in primary AML cells.

Thus, a STAT5a/b targeting molecule in combination with other therapeutic agents as well as imatinib/dastinib could be used in leukemia.

Role in breast cancer

The STAT5 proteins are not only critical in hematopeoitic malignancies, but also in several other cancers and tumours. It presumably plays complex opposing roles in breast cancer (BC) and summarised below are the studies in breast cancer cell lines, animal models of breast cancer as well as human BC samples.

In studying the role of STAT5 in breast cancer, it is only justified to enlist that STAT5 plays an important role in proliferation, survival and terminal differentiation of the mammary gland [95, 136, 137]. These functions are made possible by upregulating the pro-survival genes (bcl-xl), as well as genes for proteins found in milk, beta-casein and whey acidic protein [95, 138, 139]. STAT5 is a promoter of tumorigeneis in rodent mammary gland and the presence of STAT5a is required for the development of oncogene-induced mammary cancers in mice [140-142]. Knockout studies on mice indicate that a loss of STAT5a delays tumorigenesis and hemizygous loss of the STAT5a allele led to a delay in tumours initiation and formation [141, 142]. Additionally, constitutive activation of STAT5a or over expression of STAT5 led to the mammary tumours within 8-12 months [143]. In this study, it was noted that truncated STAT5 (carboxy terminal truncation) overexpression led to a poorly differentiated mammary tumour. Thus, it could be speculated that the poor differentiation was due to the wtSTAT5 being inhibited and that the carboxy terminal truncated STAT5 plays an important role in malignancy and tumour formation. Again, Vafaizadeh et al, using a lentiviral infection system, created a constitutively active STAT5 (cS5-F) which upon expression, led to

epithelial hyperproliferation and ER+ PR+ adenocarcinomas [144]. Nonetheless, STAT5 also has a protective role in BC cells and although STAT5B is constitutively active in human BC cell lines, over expressing a dominant negative variant of STAT5 leads to an induction of apoptosis in luminal BC cell lines [145]. Recent work by Caffarel et al, using the constitutive active JAK2-V617F mutant and expressing it in MCF-7 BC cells, led to a more invasive form of BC in xenografts [146].

Thus, while knockout studies on mice and BC cell lines indicate that a loss of STAT5 delays tumorigenesis, this is not the case in humans. Infact, one of the most notable findings from clinical studies was done using immunohistochemistry to the tyrosine phorpshoryakted protein and it was observed that while 40% of all breast cancers displayed activation of STAT3 alone, only 7% of them showed STAT5 activation. Of the tumors that presented with activated STAT5, more than 80% had activation of STAT3 as well. Furthermore, an analysis of the pathological features of these tumors revealed that cancers displaying activation of STAT5 were more likely to be highly differentiated, low grade tumors. Additionally, they were less likely to form metastases to lymph nodes. All of these properties of tumors with activation of both STAT3 and STAT5 reflected a more favorable clinical outcome than tumors displaying activation of STAT3 alone. Indeed, patient survival was significantly prolonged when tumors displayed gene expression signatures consistent with activation of STAT3 and STAT5 rather than STAT3 activation alone [147]. In another study by Yamashita et al, STAT5 was defined as a strong prognostic molecular marker in ER-positive breast cancer. In this study, they studied the expression of STAT3 and STAT5 in more than 500 breast cancer tissues by immunohistochemical techniques and observed that in ER positive patients having Stat5 positive tumours had significantly increased overall survival, thereby suggesting that expression of Stat5 is helpful in selecting patients who could possibly benefit from endocrine therapy [148]. These findings thus correspond to the notion that normally STAT5 promotes differentiation of mammary epithelium. Moreover, they also suggest that tumors with activation of STAT5 (or both STAT3 and STAT5) may be more susceptible to cell death induced by chemotherapeutic agents, which could also contribute to a more favorable prognosis. Various other studies go onto define the protective role of STAT5 in breast cancer pathogenesis. STAT5 expression and activation when studied in breast cancer samples showed a gradual loss of STAT5 activity during cancer progression [149]. Activated STAT5 was associated with a favorable prognosis in breast cancer patients [150]. Peck et al showed that a loss of pSTAT5 from the nucleus predicts for a poor clinical outcome along

with the possibility of failure to respond to endocrine therapy in ER+ve breast cancer patients [151]. Interestingly, the expression of Bcl6 is inhibited by prolactin mediated activation of STAT5 in breast cancer [152]. Again, it has been shown that the STAT3 and STAT5 signaling pathway is integrally involved in endocrine resistance and more so in the growth factor-stimulated cases [153].

Thus, there appears to be a fine interplay of functional roles between STAT3 and STAT5 in the various breast cancer subtypes. STAT5 expression corresponds to better prognosis for patient survival by an increase in epithelial cell differentiation and delaying metastasis [154].

Another aspect of STAT5 biology concerns its specificity for a specific breast cancer subtype and its role in endocrine resistance. In premenopausal women which are predominantly hormone responsive tumors or ER+ve tumors, increase in prolaction was associated with increased cancer rick [155]. STAT5 was found to be constitutively activated in ER+ ve tumours [156], and mice studies went onto demonstrate that CA-STAT5 led to the development of tumors in the mammary gland [157].

In 2012, Adrian Britschgi et al demostarted the fine cross talk between signalling pathways and that suppressing the PI3K/mTor pathway, led to an activation of the JAK2/STAT5 pathway and circumvented the efficancy of the PI3K/mTor inhibition [158]. However, dual inhibition of the PI3K/mTor pathway led to a robust response with cancer cell apoptosis and reduction in tumor growth, leading to an increase in the overall survival. This strategy of combined targeting is thus a strategy to combat triple-negative breast cancer (which is characterized by the absence of expression of estrogen and progesterone as well as ERBB2/HER2 receptors), a particularly aggressive and currently incurable disease.

There remains no doubt with the conflicting studies in mice, BC cell lines and human tumour samples, that STAT5 plays a complex role in breast cancer. It appears to have a dual role dependant on the stage of tumour preogression- promoting tumour progression in the early stages of tumour formation while inhibiting their potential to metastasize. It could also be argued that STAT5A/B plays conflicting roles in human and mouse cancer tumorigenesis. Tang et al, on a study in BC cell lines, have reported the differential activity of STAT5a and STAT5b in inducing survival and inbiting cell migration [159].

Additionally, new mouse models need to be created and studied to delineate the role of STAT5 in BC. There is now a demand to create models where STAT5 could be knocked out or overexpressed in the tumour once they are formed, so as to help study its role in later stages of cancer progression. Another caveat to studies in breast cancer lies in the various subtypes of breast cancer and the complexity of the developmental process involved in the mammary gland. The role of STAT5 is thus context specific and targeting STAT5 could not only be beneficial in a subset of the breast cancer cell types but also on the stage of the tumour development/progression and as a combination drug.

Conclusion and future prospects

The JAK-STAT5 pathway has been associated with proliferation, survival, differentiation as well as apoptosis. Activation of STAT5 is dependent on the phosphorylation, acetylation, SUMoylation as well as ubiquitination states. The importance of tetramerization for normal immune functions is well understood and several cancers have shown a dependence on the cancer-promoting actions of STAT5.

While hematopoietic malignancies have been studied in some detail, it is the role of STAT5 in solid cancers and tumours, its regulation of the immune system and the anti-tumour response elicited which warrants further research. In brief, STAT5, by expanding regulatory T cells which promote tumours and cancers by inhibiting anti-tumour activity, leads to the progression of cancers and tumours. This very role of STAT5 could be exploited for its use as a therapeutic target.

Indeed, several small molecule drugs targeting the cytokine signalling as well as JAK inhibitors have shown promise. Discovery of small molecule therapeutic drugs to target

STAT5 are in progress, although a STAT5 specific inhibitor is not yet available for use in clinical trials. The challenge to effectively target the JAK-STAT5 pathway for cancer therapy remains and needs extensive research and study.

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Table 1: Effector molecules and the kinases involved in signalling of STAT5

Ligands	JAK's and non JAK kinases
IL-2	JAK1, JAK3, Fyn, Lck, Hck, Tec,
IL-3	JAK2, Fyn, Hck, Lyn
IL-4	JAK1, JAK3
IL-5	JAK2, Btk
IL-7	JAK1, JAK3, Lyn
IL-9	JAK3
IL-10	JAK1, Tyk2
IL-15	JAK1, Lck
IL-21	JAK3
IL-22	JAK1, Tyk2
IL-27	JAK1, JAK2, Tyk2
EGF	JAK1, EGFR, Src
EPO	JAK2, Src Family
GH	JAK2, Src Family
GM-CSF	JAK2, Hck, Lyn
Insulin	JAK2, IR, Src
Leptin	JAK2
PDGF	JAK1, PDGFR, Src
PRL	JAK2, Src
TPO	Tyk2, JAK2, Lyn

Abbreviations: EGF, epidermal growth factor; EPO, erythropoietin; GH, growth hormone; GM-CSF, granulocyte macrophage-colony stimulating factor); PDGF, platelet-derived growth factor; PRL, prolactin; TPO, thrombopoietin; IL, interleukin; JAK, Janus kinase; STAT, signal transduction and activation of transcription; TYK, tyrosine kinase. Compiled from [161-163].

Table 2: Protein interaction partners of STAT5

Protein	Interacting domain
BRCA1 and BRCA2	not determined
Caveolin	not determined
CBP/p300	transactivation domain of STAT5a
CPAP	SH2 domain of STAT5
CrkL	SH2 domain of CrkL
ERK	transactivation domain of STAT5a
ERα	transactivation domain of STAT5
Ligand/CypB	N terminus of STAT5
NcoA-1/SRC-1	transactivation domain of STAT5a
Nmi	coiled-coil domain of STAT5
Oct1	transactivation domain of STAT5
p100	transactivation domain of STAT5
SMRT	coiled-coil domain of STAT5
STAP-2/BKS	transactivation domain of STAT5
TRβ1	non-determined

References: [164]